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Research Article

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# Parametric Optimization of Extracellular Chitin Deacetylase Production by *Scopulariopsis brevicaulis*

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## Abstract

The culture medium, fermentation conditions and effects of different inorganic salts on producing extracellular chitin deacetylase by *Scopulariopsis brevicaulis* were optimized. The highest enzymatic activity of deacetylation of chitin was 36 units/mL for optimum culture medium which included 2% (w/v) 3,6-*O*-carboxymethylchitin, 1% sucrose, 0.2% $NaNO_3$, 0.1% $K_2HPO_4$, 0.05% KCl, 0.4% peptone, 0.05% $MgSO_4$, 0.05% $MnSO_4$, 0.03% $CoCl_2$ and optimum cultural conditions included temperature, pH, agitation speed, fermenting time and volume and were 29°C, 7.0, 200 rmp $min^{-1}$, 96 hours and 80 mL in 300 mL Erlenmeyer flask, respectively. The capacity for producing chitin deacetylase was increased 76%, 70% and 68% when 0.05% (w/v) $MnSO_4$, 0.03% (w/v) $CoCl_2$ and 0.05% (w/v) $MgSO_4$ were added to culture solution, respectively.

**Keywords**

Chitin; Extracellular chitin deacetylase; Parametric optimization; *Scopulariopsis brevicaulis*

## Introduction

Chitin is a linear homopolymer of (1→4)-linked-N-acetyl-β-D-glucosamine, which can be easily isolated from shellfish waste. However, chitin is extremely insoluble, but can be converted to more-soluble chitosan by deacetylation. Chitosan is soluble in acid solutions, and has a wide range of uses, eg. as antimicrobial material [1], biodegradable packaging films [2], base for cosmetics [3] or material of medicine [4]. The traditional method of extracting chitin involves treatment with strong alkali at high temperatures and this is a way to remove the acetyl groups to convert it into chitosan. The process was environmentally unsafe and not easily controlled, leading to a broad and heterogeneous range of product [5].

The use of chitin deacetylase for preparation of chitosan can overcome most of these disadvantages. Chitin deacetylase (CDA, EC 3.5.1.41) [6], an enzyme that catalyzes the conversion of chitin to chitosan by the deacetylation of N-acetyl-D-glucosamine residues, has been identified in many bacteria [7], fungi [8-15], insect species [16] and Crustacea [17]. A previously unknown bacterium which had been isolated from municipal sewage can also deacetylate chitin to chitosan [18]. However, all these microorganisms had low ability to produce chitin deacetylase; activity range of pure enzyme was 0.219-35 $U \cdot mg^{-1}$. Most chitin deacetylases from these microorganisms were intracellular enzymes and purification of intracellular chitin deacetylases was difficult. The yield rates and enzymatic activities were very low, 4-29% and 2-12 $U \cdot mg^{-1}$ pure enzyme, respectively. Thus to facilitate more efficient enzymic deacetylation of chitin, it was very important to select a microorganism that could produce extracellular chitin deacetylase with high yield rate and enzymatic activity, and to research the best conditions for producing chitin deacetylase. Recently, a microorganism was isolated from soil samples collected around a chitin production factory and through micromorphology, colony and physiological, and biochemical characterization the microorganism was *Scopulariopsis brevicaulis*. It can produce extracellular chitin deacetylase with enzymatic activities in culture solution of 10-11 units $mL^{-1}$. Purification and characterization of this enzyme has been researched [14].

In this study, the parametric optimization of *S. brevicaulis* to produce extracellular chitin deacetylase is reported. Incubation conditions and effects of some metal cations on the capacity of producing chitin deacetylase were investigated.

## Materials and Methods

### Materials

Shrimp crystalline chitin was purchased from Jinan Haidebei Marine Bioengineering Co.Ltd (Jinan, China). Hexa-N-acetylchitohexaose was purchased from Sigma Chemical Co. (St. Louis, USA). Glucosamine·HCl was purchased from Zhejiang Aoxing Biotechnology Co. Ltd (Taizhou, China). Colloidal chitin, the water-soluble chitosan (54% deacetylated degree with an average molecular weight of 280 kDa) and 3,6-*O*-carboxymethylchitin (10% deacetylated degree, 0.8 degree of substitution) were prepared in the laboratory, based on the literature respectively [19-21]. All other chemicals used were commercial products of analytical grade.

### Optimizing of medium

Optimizing of medium was carried out using a minimal synthetic medium (MSM) containing: 0.2% $NaNO_3$, 0.1% $K_2HPO_4$, 0.05% KCl, inducing substrate, carbon source, nitrogen source and inorganic salts. The optimum of inducing substrate and it's dosage were chosen from four inducing substrates (chitin, colloidal chitin, 3,6-*O*-carboxymethylchitin and water-soluble chitosan) and four different dosage of every inducing substrate (the dosage of every inducing substrate were 0.5% (w/v), 1% (w/v), 1.5% (w/v) and 2.0% (w/v) respectively). The optimum of carbon source and it's dosage were chosen from five carbon sources (glucose, sucrose, lactose, maltose, glucosamine) and four difference dosage of every carbon source (the dosage of every carbon source were 0.6% (w/v), 0.8% (w/v), 1.0% (w/v) and 1.2% (w/v), respectively.). The optimum of nitrogen source and it's dosage were chosen from six nitrogen sources (peptone, beef extract, yeast extract, $NH_4NO_3$, $NH_4Cl$, $(NH_4)_2SO_4$)

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and three difference dosage of every nitrogen source (the dosage of every nitrogen source was 0.2% (w/v), 0.4% (w/v) and 0.6% (w/v) respectively.), and optimum of inorganic salts and their dosage were chosen from eight inorganic salts ($MgSO_4$, $CaCl_2$, $ZnSO_4$, $CuSO_4$, $MnSO_4$, $CoCl_2$, $FeCl_3$, $FeCl_2$) and four different dosage of every inorganic salt were 0.01% (w/v), 0.03% (w/v), 0.05% (w/v) and 0.07% (w/v) respectively.). In the process of optimizing medium, all culture conditions were 100 mL of the medium in a 300 mL Erlenmeyer flask, aerobically cultured at 29°C for 96 hours on a rotary shaker (200 rpm $min^{-1}$). After centrifugation with 8000×g at 4°C for 30 min, the supernatant was used as bioassay. After the optimal culture medium composition was determined, the culture conditions (including temperature, pH, agitation speed, fermenting time and volume) were optimized again.

### Effect of culture conditions

With the use of the optimal culture medium composition, the effect of different initial pH (5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0), temperature ( 25°C, 27°C, 29°C, 31°C, 33°C, 35°C, 37°C), cultivation volume (40 mL, 60 mL, 80 mL, 100 mL in 300 mL Erlenmeyer flask), agitation speed (120, 140, 160, 180, 200, 220, 240, 260 rpm $min^{-1}$) and cultivation time (0, 6, 12, 18, 24, 36, 48, 60, 72, 84, 96, 108, 120, 132 hours) on the mycelium weight and the production of chitin deacetylase were investigated by fermenting in 300 mL Erlenmeyer flask through rotary shaker until the optimal culture condition was found.

### Analytical methods

The concentration of cultivated mycelia was determined by centrifuging the fermented broth; washing the precipitate three times using three times volume distilled water to precipitate, and drying at 80°C to constant weight.

The chitin deacetylase enzyme activity assay based on the literature method [22-24] was performed in glass tubes using 50 µL 50 mM Tris–HCl buffer pH 7.5, 100 µg hexa-N-acetylchitohexaose in 100 µL water, and 50 µL enzyme preparations. Incubation time was 15 min at 55°C, and the reaction was terminated by the addition of 250 µL 5% (w/v) $KHSO_4$.

For color formation, 250 µL 5% w/v $NaNO_2$ was added, and the tubes capped immediately and allowed to stand with occasional shaking for 15 min, and 250 µL 12.5% w/v aqueous 3-methy1-2-benzothiazolinonehydrazone hydrochloride (freshly prepared each day) was added and the mixture was heated at 100°C for 3 min. After cooling to room temperature, 250 µL 0.5% w/v $FeCl_3$ was added and the developing color was read after 30 min at 650 nm. Standard curves were prepared with D-glucosamine·HCl standard.

Units of enzyme activity were estimated by using Hexa-Nacetylchitohexaose (166 nmol) as substrate in 50 µL 50 mM Tris–HCl buffer pH 7.5. Incubation time was 15 min at 55°C (that it is the optimum temperature of this chitin deacetylase), and the reaction was terminated by the addition of 250 µL 5% w/v $KHSO_4$.

One unit of chitin deacetylase activity is defined as the amount of the enzyme required to produce 1 mmol of acetate/min. when incubated with hexa-N-acetylchitohexaose as described above.

## Results and Discussion

### Optimizing of medium

**The effect of chitin or chitin derivative on the production of chitin deacetylase:** When temperature, initial pH, agitation speed, fermenting time and volume were 29°C, 6.5, 200 rmp $min^{-1}$, 96 hours and 100 mL in 30 mL Erlenmeyer flask, respectively, a series of experiments were carried out to study the effect of chitin or chitin derivative and their concentration on the chitin deacetylase production. The results showed that the activities of producing chitin deacetylase were in the following order: 3,6-*O*-carboxymethylchitin>water-soluble chitosan>colloidal chitin>chitin. The maximal chitin deacetylase activity was obtained when the concentration of 3,6-di-*O*-carboxymethylchitin was 2% (Figure 1).

**The effect of carbon source on the production of chitin deacetylase:** To study the effect of carbon sources on the production of chitin deacetylase, culture was carried out in 3,6-di-*O*-carboxymethylchitin medium (0.2% $NaNO_3$, 0.1% $K_2HPO_4$, 0.05% KCl, 2% 3,6-di-*O*-carboxymethylchitin) either containing no additional carbon source or glucose or sucrose or lactose or maltose or glucosamine, respectively. The production of chitin deacetylase by *S. brevicacclis* was greatly enhanced by the addition of sucrose into the medium and 1% sucrose was most effective for chitin deacetylase production. For the same concentration (1%) of carbon source, the results showed the activities of producing chitin deacetylases were in the following order: sucrose>glucose>glucosamine >maltose>nothing >lactose.

**The effect of nitrogen source on the production of chitin deacetylase:** The effect of different nitrogen sources on the chitin deacetylase production was tested in a medium (0.2% $NaNO_3$, 0.1% $K_2HPO_4$, 0.05% KCl, 2% 3,6-di-*O*-carboxymethylchitin, 1% sucrose) containing peptone, beef extract, yeast extract, $NH_4NO_3$, $NH_4Cl$ and $(NH_4)_2SO_4$, respectively. The results showed that the organic nitrogen source (including peptone, beef extract, yeast extract) rather than the inorganic nitrogen source (including $NH_4NO_3$, $NH_4Cl$, $(NH_4)_2SO_4$) was more suitable for chitin deacetylase production by *S. brevicaulis*. Among the organic nitrogen source, peptone was the best organic source to produce chitin deacetylase by *S. brevicaulis*. It was found that 0.4% peptone was most effective for chitin deacetylase production. For the same concentration (0.4%) of organic source, the results showed the activities of producing chitin deacetylases were in the following order: peptone>yeast extract>beef extract>nothing.

**The effect of inorganic salt on the production of chitin deacetylase:** The effect of different inorganic salts on the chitin

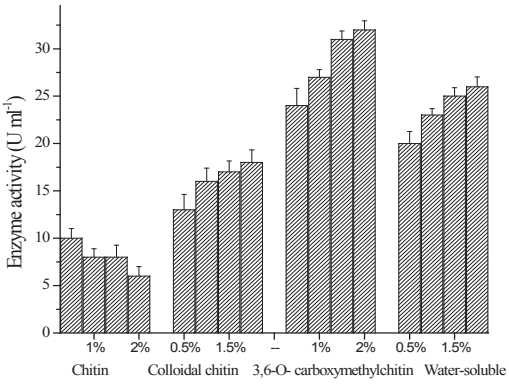


**Figure 1:** Effect of the chitin or chitin derivative and their concentrations on chitin deacetylase production from *Scopulariopsis brevicaulis*. Results are expressed as means ± S.D. (n=3).

deacetylase production was tested in a medium (0.2% $NaNO_3$, 0.1% $K_2HPO_4$, 0.05% KCl, 2% 3,6-di-*O*-carboxymethylchitin, 1% sucrose, 0.4% peptone) containing $MgSO_4$, $CaCl_2$, $ZnSO_4$, $CuSO_4$, $MnSO_4$, $CoCl_2$, $FeCl_3$, $FeCl_2$ (metal ions concentration range 0.01% to 0.07%), (Table 1). The capacity of *S. brevicaulis* for producing chitin deacetylase was increased by $Mn^{2+}$, $Zn^{2+}$, $Mg^{2+}$, $Co^{2+}$. The $Mn^{2+}$, $Co^{2+}$ and $Mg^{2+}$ are very strong activators. When 0.05% $MnSO_4$ or 0.03% $CoCl_2$ or 0.05% $MgSO_4$ were added to culture solution, the capacity of producing chitin deacetylase was increased 76%, 70% and 68%, respectively. We had researched their combined effect. Results showed that the capacity of producing chitin deacetylase of *S. brevicaulis* were increased 75%, 71%, 74% and 75%, when 0.05% $MnSO_4$ and 0.03% $CoCl_2$, 0.03% $CoCl_2$ and 0.05% $MgSO_4$, 0.05% $MnSO_4$ and 0.05% $MgSO_4$, 0.05% $MnSO_4$ and 0.03% $CoCl_2$ and 0.05% $MgSO_4$ were combined to add to medium respectively. In contrast, $Fe^{3+}$ led to completely inhibition of the capacity to produce chitin deacetylase. $Fe^{2+}$ and $Cu^{2+}$ only led to partial inhibition of the capacity to produce chitin deacetylase (Table 1).

## Effect of culture conditions

**The effect of initial pH on the production of chitin deacetylase:** When fermenting temperature, cultivation time, rotation speed and volume in 300 mL Erlenmeyer flask were 29°C, 96 hours, 200 rpm $min^{-1}$ and 100 mL, respectively, investigation of the effect of initial pH on the production of chitin deacetylase and mycelium weight using the optimal culture medium (2% w/v 3,6-*O*-carboxymethylchitin, 1% sucrose, 0.2% $NaNO_3$, 0.1% $K_2HPO_4$, 0.05% KCl, 0.4% peptone, 0.05% $MgSO_4$, 0.05% $MnSO_4$, 0.03% $CoCl_2$) showed that pH had obvious effect on the production of chitin deacetylase activity and mycelium weight (Figure 2). When the initial pH was 7.0, chitin deacetylase activity was highest, 34 $U{\cdot}mL^{-1}$. However, highest mycelium weight was obtained when the initial pH was 6.5. We selected the initial pH 7.0 (Figure 2).

**The effect of temperature on the production of chitin deacetylase:** When initial pH, cultivation time, rotation speed and volume in 300 mL Erlenmeyer flask were 7.0, 96 hours, 200 rpm $min^{-1}$ and 100 mL respectively, investigation of the effect of temperature on the production of chitin deacetylase and mycelium weight using the optimal culture medium showed that *S. brevicaulis* produced highest chitin deacetylase activity and mycelium weight when temperature was 29°C and 27°C, respectively (Figure 3). We selected the temperature 29°C.

**The effect of medium volume on the production of chitin deacetylase:** Investigation of the effect of the medium volume in 300 mL Erlenmeyer flask and the rotation speed on the production of chitin deacetylase and mycelium weight using the optimal culture medium showed that the optimal medium volume was 80 mL in 300 mL Erlenmeyer flask, About medium volume in 300 mL Erlenmeyer flask, the results showed the activities of producing chitin deacetylases were in the following order: 80 mL >60 mL>40 mL>100 mL (data no shown). The mycelium weight increased gradually with the rotation speed from 120 rpm to 260 rpm. However, when the rotation speed was 200 rpm, the enzymatic activity was highest, 35 U/mL (Figure 4).

**The effect of cultivation time on the production of chitin deacetylase:** To study the effect of cultivation time on the production of chitin deacetylase and mycelium weight, incubation was carried out in the optimal 80 mL medium (2% (w/v) 3,6-*O*-carboxymethylchitin, 1%sucrose, 0.2% $NaNO_3$, 0.1% $K_2HPO_4$, 0.05% KCl, 0.4% peptone,

**Table 1:** Effect of metal ions on producing chitin deacetylase capacity of *Scopulariopsis brevicaulis*.

| Metal ions | Concentration (%) | Relative chitin deacetylase activity [a] |
|---|---|---|
| None | | 100 |
| $CaCl_2$ | 0.01 | 100 |
| | 0.03 | 101 |
| | 0.05 | 101 |
| | 0.07 | 102 |
| $MnSO_4$ | 0.01 | 113 |
| | 0.03 | 147 |
| | 0.05 | 176 |
| | 0.07 | 179 |
| $ZnSO_4$ | 0.01 | 105 |
| | 0.03 | 121 |
| | 0.05 | 127 |
| | 0.07 | 128 |
| $MgSO_4$ | 0.01 | 126 |
| | 0.03 | 149 |
| | 0.05 | 168 |
| | 0.07 | 171 |
| $CoCl_2$ | 0.01 | 147 |
| | 0.03 | 170 |
| | 0.05 | 172 |
| | 0.07 | 167 |
| $FeCl_2$ | 0.01 | 64 |
| | 0.03 | 32 |
| | 0.05 | 29 |
| | 0.07 | 13 |
| $FeCl_3$ | 0.01 | 0 |
| | 0.03 | 0 |
| | 0.05 | 0 |
| | 0.07 | 0 |
| $CuSO_4$ | 0.01 | 43 |
| | 0.03 | 25 |
| | 0.05 | 13 |
| | 0.07 | 6 |

[a] The activities were assayed under the standard conditions and expressed as a percentage of the activity in the absence of compound

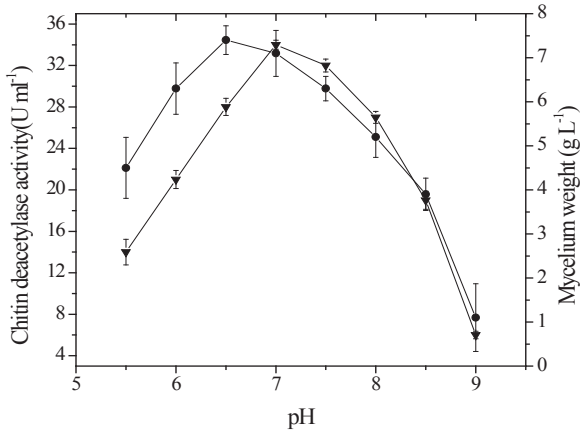


**Figure 2:** Effect of initial pH on the mycelium formation (●) and chitin deacetylase production (▼) by *Scopulariopsis brevicaulis*. Results are expressed as means ± S.D. (n=3).

0.05% $MgSO_4$, 0.05% $MnSO_4$, 0.03% $CoCl_2$) at pH 7.0, culture temperature and rotation speed were 29°C and 200 rpm, respectively. *S. brevicaulis* produced highest chitin deacetylase activity when incubation time was 96 hours. The exponential growth phase of *S. brevicaulis* was 12-72 hours (Figure 5).

In summary, the production of chitin deacetylase and mycelium weight was strongly dependent on the culture conditions. Optimum medium contents and culture conditions for producing extracellular chitin deacetylase from S. brevicaulis are shown in table 2. The highest extracellular chitin deacetylase activity was 36 U/mL under the conditions. It was higher than the extracellular chitin deacetylase activity (2.47 U/mL) produced by *Colletotrichum lindemuthianum* [25] (Table 2).

## Conclusions

A novel strain belonging to *S. brevicaulis* which produced extracellular chitin deacetylase has been selected from some soils and sludge samples. The optimum medium contents and cultural conditions for chitin deacetylase activity of the strain were determined. Compared with all other corresponding microorganism to produce extracellular chitin deacetylase report, most of literatures only reported pure enzyme activity, the extracellular chitin deacetylase activity from *Colletotrichum lindemuthianum* [25] was reported, but it is lower than *S. brevicaulis*. *S. brevicaulis* had higher capacity to produce extracellular chitin deacetylase and the capacity was activated by $Mn^{2+}$, $Zn^{2+}$, $Mg^{2+}$and $Co^{2+}$.

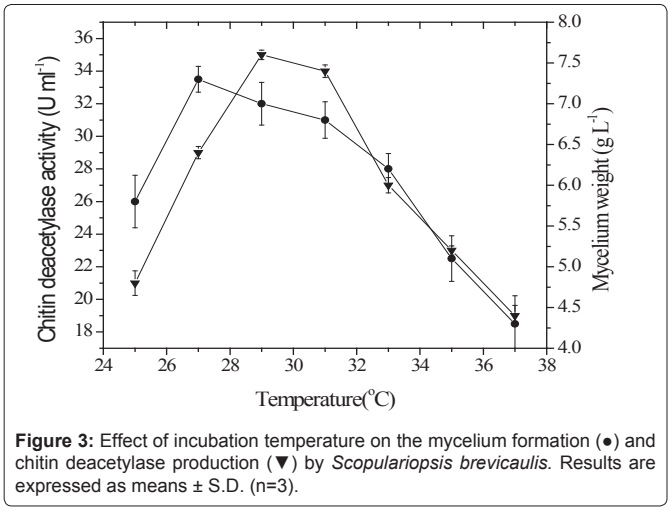


**Figure 3:** Effect of incubation temperature on the mycelium formation (●) and chitin deacetylase production (▼) by *Scopulariopsis brevicaulis*. Results are expressed as means ± S.D. (n=3).

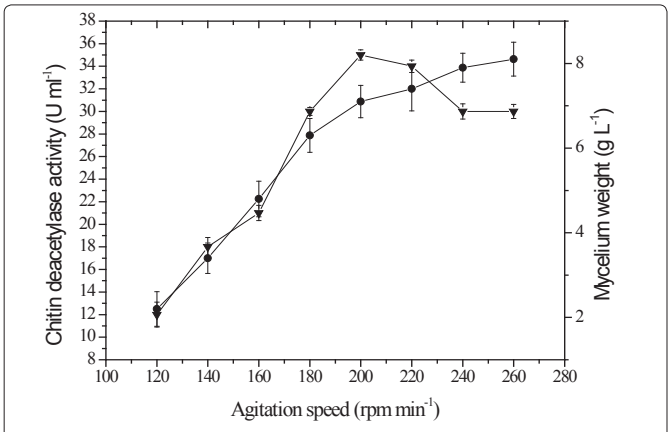


**Figure 4:** Effect of agitation speed on the mycelium formation (●) and chitin deacetylase production (▼) by *Scopulariopsis brevicaulis*. Results are expressed as means ± S.D. (n=3).

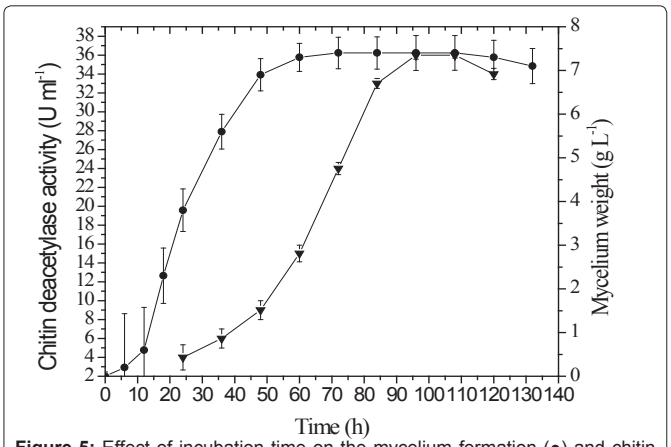


**Figure 5:** Effect of incubation time on the mycelium formation (●) and chitin deacetylase production (▼) by *Scopulariopsis brevicaulis*. Results are expressed as means ± S.D. (n=3).

**Table 2:** The optimum medium contents and cultural conditions for chitin deacetylase activity of *Scopulariopsis brevicaulis*.

| Optimum culture medium | | Optimum cultural condition | |
|---|---|---|---|
| **Component** | **Concentration (g/100 mL)** | **Factor** | **Value** |
| 3,6-*Di*-*O*-carboxy-methylchitin | 2 | pH | 7.0 |
| Sucrose | 1 | Temperature | 29°C |
| Peptone | 0.4 | Volume | 80 mL in 300 mL Erlenmeyer flask |
| $NaNO_3$ | 0.2 | Time | 96 hours |
| $K_2HPO_4$ | 0.1 | Agitation speed | 200 rmp $min^{-1}$ |
| KCl | 0.05 | | |
| $MgSO_4$ | 0.05 | | |
| $MnSO_4$ | 0.05 | | |
| $CoCl_2$ | 0.03 | | |
| **Yields from Optimised culture** | | | |
| Chitin deacetylase activity | 36 ± 0.4 U/mL[a] | Biomass yield | 7.0 ± 0.6 g/L[a] |

[a]Results are expressed as means ± S.D. (n=3).

### Acknowledgement

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